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A novel *in vivo* model to study bacterial pathogenesis and screen potential therapeutic targets

Escherichia coli sepsis and meningitis are among the leading causes of neonatal bacterial infections, affecting from 0.5 to 5 per 10 000 live births in developed countries, with far greater numbers in developing countries. *E. coli* K-1 meningitis alone contributes to >50 000 deaths per year worldwide (Gladstone *et al.*, 1990; de Louvois *et al.*, 1991; Kim, 2003; Alarcon *et al.*, 2004; Bonacorsi & Bingen, 2005). As *E. coli* K-1 meningitis is limited mostly to neonates, who depend largely on an innate immune response to counter infection, we previously hypothesized that insects are useful models to study the pathogenesis of *E. coli* K-1. For the first time, we demonstrated that locusts can be used as a model to study *E. coli* K-1 pathogenesis (Khan & Goldsworthy, 2007; Mokri-Moayyed *et al.*, 2008). The significant findings included the fact that *E. coli* K-1 invades the locust central nervous system and kills locusts. Furthermore, *E. coli* K-1 pathogenesis within both locust and vertebrate systems was shown to be dependent upon several common established virulence factors (Khan & Goldsworthy, 2007; Mokri-Moayyed *et al.*, 2008). Such data support the use of a locust system to successfully model *E. coli* infection and study its pathogenesis. In the present study, it was determined further whether locusts can be used as an *in vivo* model to study bacterial pathogenesis and to screen potential vaccine targets against *E. coli* K-1 infection.

African migratory locusts, *Locusta migratoria*, between 15 and 30 days old with a fully developed innate immune system were used in the present study (Goldsworthy *et al.*, 2003). They were fed on bran, wheat seedlings and fresh grass and live an average of 80–100 days. *E. coli* K-1 strain RS218 (O18 : K-1 : H7) is a spontaneous rifampicin-resistant mutant derived from the cerebrospinal fluid of a neonate with meningitis (Khan & Goldsworthy, 2007; Achtman *et al.*, 1983). In addition, *Staphylococcus aureus* (clinical isolate obtained from blood cultures provided by S. Alsam, Luton & Dunstable Hospitals, UK) was used. The sensitivity patterns of *S. aureus* demonstrated its susceptibility to gentamicin and resistance to penicillin, *in vitro*. In addition, a laboratory strain of *E. coli* K-12 strain HB101 was used as a non-invasive isolate. All bacterial isolates were grown at 37 °C in Luria–Bertani broth. A clinical isolate of *Acanthamoeba castellanii* of the T4 genotype was used (ATCC 50492) by growing without shaking in 10 ml PYG culture medium [0.75 % (w/v) proteose peptone, 0.75 % (w/v) yeast extract and 1.5 % (w/v) glucose] in T-75 tissue culture flasks at 30 °C as described previously (Khan & Siddiqui, 2009). The medium was refreshed 24 h prior to experiments, which resulted in more than 95 % amoebae in the infective trophozoite stage.

For all assays, locusts were allocated randomly into groups of ten, except where indicated otherwise, and experiments were repeated at least three times. For mortality assays, each experimental locust was injected with 20 µl culture medium containing 2×10^6 bacterial c.f.u. into the haemocoel of the locust abdomen by inserting the needle into the intersegmental membrane between two abdominal terga as previously described, and mortality was recorded every 24 h. In the control group, locusts were injected with culture medium alone.

In some experiments, locusts were injected with a variable number of heat-killed bacteria or *A. castellanii* (heat-kill was achieved by incubating at 65 °C for 30 min and confirmed by culturing). For simplicity, this is described as 'immunization', although in general invertebrates are not known to possess an acquired immune system. So-called 'immunized' locusts were kept for 24 h at 30 °C. Following this incubation, locusts were challenged with live invasive *E. coli* K-1 (2×10^6 c.f.u.) and mortality was recorded every 24 h. The control group was injected with culture medium alone at both time intervals. For some experiments, locusts were divided into seven groups and injected with heat-killed bacteria (10^6 c.f.u.). Next, each group was challenged with live invasive *E. coli* K-1 (2×10^6 c.f.u.) every 24 h up to 7 days and mortality was recorded.

Heat-killed non-invasive *E. coli* K-12 can protect locusts against subsequent challenge of invasive K-1

Consistent with previous findings, locusts injected with *E. coli* K-1 alone (2×10^6 c.f.u.) showed 100 % mortality within 72 h, while K-12 showed zero mortality. To determine whether 'immunization' with heat-killed K-1 (10^6 c.f.u.) or K-12 (10^6 c.f.u.) can protect locusts against subsequent challenge with live K-1 (2×10^6 c.f.u.), assays were performed as described above. It was surprising to note that locusts immunized with either heat-killed K-1 or heat-killed K-12 showed zero mortality with subsequent injection of live K-1 within 72 h. Experiments were repeated three times with reproducible results. The control group of locusts that were injected with culture medium alone showed 0 % mortality in 72 h. Similarly, locusts injected with heat-killed bacteria without subsequent challenge with live K-1 showed zero mortality, indicating no adverse effects due to so-called 'immunization'. The results revealed that prior injection with heat-killed *E. coli* K-12 can indeed protect locusts against the consequent challenge of infection with K-1. The use of non-invasive *E. coli* K-12, safely, to protect a host against an invasive strain was unexpected. A PubMed literature search revealed no evidence of any such studies carried out in vertebrates and warrants further investigations.

Use of different inoculum of heat-killed *E. coli* K-12 to 'immunize' locusts

To determine the effects of the inoculum size on immunization, locusts were injected

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with 10^3–10^6 c.f.u. of heat-killed *E. coli* K-12, left for 24 h and then injected with live K-1 (2×10^6 c.f.u.). One hundred per cent locust mortality occurred in groups that were immunized with 10^3 c.f.u. of heat-killed K-12 in 72 h. In locusts immunized with 10^5 c.f.u. of K-12, 16.6 % ± 2.2 mortality was observed within 72 h. However, in the group of locusts injected with 10^4 c.f.u. of heat-killed *E. coli* K-12, 83 % ± 7.4 mortality was observed, revealing that inoculum size has an effect on the immunization of the locusts. In contrast, locusts injected with 5×10^5 and 10^6 c.f.u. of heat-killed *E. coli* K-12 alone showed 0 % mortality within 72 h. Again, there was no mortality in the control group (culture medium-injected locusts). In the locusts group injected with live K-1 (2×10^6 c.f.u.) alone, 100 % mortality was observed within 72 h with more than 50 % ± 6.1 mortality occurring within 48 h.

Use of various microbes to induce immunity against *E. coli* K-1

Several lines of evidence suggest that insects possess and/or induce antimicrobial peptides in response to microbial infection (Steiner *et al.*, 1988; Hoffmann & Hoffmann, 1990; Cociancich *et al.*, 1994; Otvos, 2000; Andrä *et al.*, 2001). To determine immune specificity, locusts were injected with heat-killed K-12 (10^6 c.f.u.), heat-killed *S. aureus* (10^6 c.f.u.) and heat-killed *Acanthamoeba* species (10^6) and left for 24 h. Following this, locusts were challenged with live K-1 (2×10^6 c.f.u.) and mortality was recorded every 24 h. The results revealed no mortality in locusts immunized with heat-killed *E. coli* K-12 within 72 h. However, locusts injected with heat-killed *S. aureus* or *Acanthamoeba* species showed >90 % mortality within 72 h, suggesting immune specificity.

E. coli K-12-induced immunity lasts up to 5 days

Insects are known to rely on innate immune system to counter infections (Steiner *et al.*, 1988; Hoffmann & Hoffmann, 1990; Cociancich *et al.*, 1994; Otvos, 2000; Andrä *et al.*, 2001). To establish how long *E. coli* K-12-induced immunity persists, locusts were divided into seven groups and injected with heat-killed bacteria (10^6 c.f.u.). Next, each group was challenged with live *E. coli* K-1 (2×10^6 c.f.u.) every 24 h up to 7 days and mortality was recorded. It was observed that locusts immunized with K-12, kept for 24 h and then challenged with live K-1 (2×10^6 c.f.u.) showed zero mortality within 72 h. Locusts injected with K-12, kept for 48 h and then challenged with live K-1 showed zero mortality and similar results were observed for up to 5 days. However, locusts injected with K-12, kept for 6 days and then challenged with live K-1 showed 80 % ± 5.4 mortality, while locusts immunized for 7 days recorded 100 % mortality. Our findings that K-12-induced immunity in locusts against invasive K-1 persists for up to 5 days are novel. Notably, recent studies have shown that cells of the innate immune system are capable of 'memory', and of mounting rapid protection, challenging previous thought that only B cells and T cells can store memory to ward off subsequent infection (Gillard *et al.*, 2011). Although the notion of 'specific memory' in invertebrates is a fascinating area that warrants further investigation, the underlying mechanisms remain unknown. It is suggested that induction of non-specific mechanisms on their own may lead to more specific memory when expressed in combination (Kurtz, 2005). Examples include Toll, immune deficiency (Imd), peptidoglycan recognition molecules and lectins, and fibrinogen-related proteins that consist of one or two Ig superfamily domains, which could be expressed stably in the host after the primary exposure (reviewed by Kurtz, 2005) and may explain the findings observed in the present study. Future studies will identify the complementary or alternative mechanisms in the invertebrate immune system that give rise to specific memory.

Usefulness of locusts as a model to screen potential therapeutic agents

At present, the recommended antibiotic against *E. coli* K-1 meningitis in clinical settings is gentamicin. To demonstrate the potency of gentamicin in our model system, locusts were injected with K-1 as described above, followed by injection of gentamicin (25 μg to achieve 100 μg ml^{-1}; as the total haemolymph in locusts is normally 250 μl) 60 min post-infection. For negative antimicrobial control groups, rifampicin-resistant K-1-infected locusts were injected with rifampicin at the above concentrations. The administration of antibiotics was continued, once a day, for up to 5 days. It was observed that gentamicin protected locusts against K-1-mediated death, i.e. mortality recorded at 7 % ± 4 after 5 days. For controls, K-1-infected locusts injected with rifampicin showed 100 % mortality, while locusts injected with antibiotics alone showed mortality at levels similar to those injected with K-12.

In an attempt to determine the usefulness of the locust model to investigate antimicrobial chemotherapy against Gram-positive bacteria, *S. aureus* was used. The sensitivity patterns of *S. aureus* demonstrated its susceptibility to gentamicin and resistance to penicillin *in vitro*, hence penicillin was used as a negative antimicrobial control. Locusts injected with 20 μl of a suspension (2×10^6 c.f.u.) of *S. aureus* showed 72.5 % ± 5 mortality. To demonstrate the potency of gentamicin, locusts were injected with *S. aureus*, followed by injection of the antibiotic as described above. For controls, bacteria-infected locusts were injected with penicillin (25 μg to achieve 100 μg ml^{-1}). Interestingly, gentamicin showed potent effects in protecting locusts against *S. aureus*-mediated mortality at aforementioned concentrations, i.e. zero mortality recorded after 5 days. The negative antimicrobial control group showed mortality at levels similar to *S. aureus*-infected groups, i.e. 65 % ± 4 mortality. Overall, these findings suggest the usefulness of locusts as an *in vivo* model to screen potential therapeutic agents.

In conclusion, these studies suggested that immunization at a high inoculum with non-invasive *E. coli* K-12 can protect locusts against invasive K-1, and the locust immune system is capable of memory and mounting protection against subsequent infection for up to 5 days. Additionally, the usefulness of locusts as a novel *in vivo* model to screen large chemical (natural or synthetic) libraries is highlighted. Future studies will determine bacterial antigenic determinants and how the innate memory functions in locusts. A complete understanding of how locusts' innate immune cells (i.e. haemocytes) respond robustly and

specifically against bacterial pathogens will be crucial for developing effective vaccines and/or exerting early control of neonatal *E. coli* infection.

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Ruqaiyyah Siddiqui,[1] Khadijo Osman[2] and Naveed Ahmed Khan[1]

[1]Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan

[2]School of Biological and Chemical Sciences, Birkbeck, University of London, UK

Correspondence: Naveed Ahmed Khan (Naveed5438@gmail.com)

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